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Determinants of incubation period: do reptilian embryos hatch after a fixed total number of heart beats?

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SUMMARY

The eggs of birds typically hatch after a fixed (but lineage-specific) cumulative number of heart beats since the initiation of incubation. Is the same true for non-avian reptiles, despite wide intraspecific variation in incubation period generated by variable nest temperatures? Non-invasive monitoring of embryo heart beat rates in one turtle species (*Pelodiscus sinensis*) and two lizards (*Bassiana duperreyi* and *Takydromus septentrionalis*) show that the total number of heart beats during embryogenesis is relatively constant over a wide range of warm incubation conditions. However, incubation at low temperatures increases the total number of heart beats required to complete embryogenesis, because the embryo spends much of its time at temperatures that require maintenance functions but that do not allow embryonic growth or differentiation. Thus, cool-incubated embryos allocate additional metabolic effort to maintenance costs. Under warm conditions, total number of heart beats thus predicts incubation period in non-avian reptiles as well as in birds (the total number of heart beats are also similar); however, under the colder nest conditions often experienced by non-avian reptiles, maintenance costs add significantly to total embryonic metabolic expenditure.

Key words: embryonic development, heart rate, metabolic rate, thermal dependence, reptile, thermal time.

INTRODUCTION

Rates of development comprise one of the most important axes of life-history variation and span an enormous range. Generation times range from minutes or hours in some bacteria, through to decades in many vertebrates (Calder, 1984). In many oviparous (egg-laying) organisms, the duration of incubation of the eggs may be a critical life-history variable. The duration of incubation relative to the rest of the lifespan can vary even within a single phylogenetic lineage; for example, some chameleons spend more than 50% of their total lifespan within the egg (Karsten et al., 2008) whereas in other lizards, the egg stage comprises <2% of the maximum lifespan [e.g. *Varanus komodoensis* (Cogger and Zweifel, 1992)]. The egg stage differs from later life-history stages in many distinctive ecological characteristics. Most notably, embryos of most species (except those with dispersive eggs) are immobile and thus are unable to disperse, thermoregulate or feed or protect themselves against predators. We might thus expect strong selection on the duration of the incubation period and on the timing of hatching (Shine, 1978; Shine and Olsson, 2003).

Despite the strong fitness consequences of intraspecific variation in the duration of incubation [and thus the timing of hatching, e.g. *Amphibolurus muricatus* (Warner and Shine, 2007)], field studies often report extensive environmentally induced variation in these traits, even within a single population (Perrins, 1967; Olsson and Shine, 1997). Some of this variation is under parental control; for example, many birds delay brooding the eggs until all have been laid, and the low temperatures of an unattended nest delay the onset of embryonic development (Stoleson and Beissinger, 1995). More generally, the thermal regime that an egg experiences can massively alter its total incubation period, especially in species without parental control of nest temperatures. For example, eggs of the lizard *Bassiana duperreyi* can hatch in 26 days if kept at 30°C but require more than 60 days if kept at 22°C (Shine and Harlow, 1996).

What factors determine the relationship between incubation temperatures and total developmental periods? The simplest explanation is that the transformation from zygote to hatchling is prolonged by cool conditions because temperature regulates the rate of chemical activities (including those contributing to overall metabolic rate and hence rates of embryogenesis). Although many additional phenomena [e.g. embryonic diapause, precocial *vs* altricial hatching (Ar and Tazawa, 1999; Andrews et al., 2008)] can also affect the duration of the egg stage, much of the variation in total incubation periods appears to be driven by variation in rates of embryogenesis. Consistently, embryos developing at high temperatures have shorter development times than those kept at low temperatures (Deeming and Ferguson, 1991; Hoegh-Guldberg and Pearse, 1995). This phenomenon is so widespread that it has been formalised by the concept of 'thermal time' [the linear relationship between development rate and temperature (Honek, 1996; Trudgill et al., 2005)].

The physiological mechanisms that link developmental time to temperature are probably complex but the cardiovascular system is known to play a critical role in nutrient and oxygen delivery during embryonic development (Birchard and Reiber, 1996; Birchard and Deeming, 2004; Tazawa, 2005). When embryonic growth is accelerated by higher temperature, the cardiovascular system must deliver nutrients at an increased rate to fuel the faster growth. Therefore, the relationship between temperature and incubation period may be driven by the thermal dependence of cardiovascular function. In keeping with this idea, the rate of embryonic heart beat (an indicator of cardiovascular activity) is positively correlated with developmental rate in birds (Ar and Tazawa, 1999; Tazawa, 2005).

Within an avian lineage, the cumulative number of heart beats between oviposition and hatching is relatively constant, consistent with the general constancy in heart beats per life span for adult endotherms (Ar and Tazawa, 1999).

Non-avian reptiles present an additional complication in this respect because embryonic development occurs over a much wider range of temperatures than in birds (Deeming and Ferguson, 1991). So, does the cumulative number of heart beats predict incubation duration in lizards and turtles (as it does in birds) despite the enormous variation in incubation periods generated by fluctuations in nest temperatures? Although previous attempts to monitor heart rates in reptile embryos have had to overcome major technical obstacles (Birchard and Deeming, 2004), recent methodological advances facilitate non-invasive monitoring (Radder and Shine, 2006; Du and Shine, 2008) and thus provide an opportunity to clarify the issues raised above. In the present study, we measured the thermal dependence of embryonic heart beat rates in three species of non-avian reptiles. With data on the duration of incubation at each of these temperatures, we could then calculate the total number of heart beats of embryos during incubation. The results of these calculations allowed us to assess whether (as in birds) we can predict the duration of reptilian incubation from the cumulative total number of heart beats since oviposition.

MATERIALS AND METHODS

Egg collection and incubation

We used three species of oviparous non-avian reptiles from divergent phylogenetic lineages: the Australian three-lined skink *Bassiana duperreyi* Wiegmann (Scincidae), the Chinese northern grass lizard *Takydromus septentrionalis* Gunther (Lacertidae) and the Chinese soft-shelled turtle *Pelodiscus sinensis* Gray (Trionychidae). In November 2007, we collected 26 freshly laid eggs of *B. duperreyi* from field nests near Canberra in southeastern Australia [see Shine and Harlow and Shine et al. (Shine and Harlow, 1996; Shine et al., 1997) for details of study area and species biology]. In May 2008, we collected 14 *T. septentrionalis* eggs laid in captivity by females recently field-collected in Zhejiang, eastern China, and obtained 35 *P. sinensis* eggs from a private turtle farm, also in Zhejiang. The mean developmental stages of embryos at oviposition [based on the classification schemes of Hubert and Tokita and Kuratani (Hubert, 1985; Tokita and Kuratani, 2001)] were stage 31, 26 and 5 for *B. duperreyi*, *T. septentrionalis* and *P. sinensis*, respectively. All eggs were weighed (±0.001 g) and individually incubated in 64 ml glass jars filled with moist vermiculite (–200 kPa). The jars were then incubated at 25°C (for *B. duperreyi* eggs) or 28°C (for *T. septentrionalis* and *P. sinensis* eggs), close to the mean temperature of natural nests in each species (Shine et al., 1997; Du, 2003; Du and Feng, 2008).

Heart rate detection

We measured heart rates of embryos approximately 25% through the total incubation period in all species. Previous studies on one of our study species (Radder and Shine, 2006) and our unpublished data on both of the other taxa (W.-G.D. and R.S., unpublished data), reveal no significant ontogenetic shift in mean heart rates from <25% to >90% of incubation (at any given temperature); thus, the exact timing of our heart rate measurements relative to embryogenesis should have little impact on the rate estimates. Heart rates [beats per minute (beats min^{-1})] were measured using an infrared heart rate monitor (Buddy system; Avian Biotech; http://www.avianbiotech.com/buddy.htm) on day 10 in *B. duperreyi*, day 7 in *T. septentrionalis* and day 15 in *P. sinensis,* respectively. All eggs were placed in incubators set at 20°, 25°, 30°, 33° or 35°C for a two hour acclimation period prior to measurement and were then placed individually on the monitor to record heart rate. The Buddy system works by shining an infrared beam onto the surface of the egg, detecting minute distortions caused by embryonic heart beats; recording heart rates generally takes less than a minute and does not affect the egg or embryo. As our measure of mean heart beat rate, we used the mean rate in the first 30 s after the monitor first gave a reliable, consistent reading (which typically occurred a few seconds after the machine was switched on but occasionally took 10 or 15 s to stabilise). The order of exposure of each egg to test temperatures was random.

Calculations of total heart beats and total effective heart beats

We used the heart rate data to develop quadratic equations that describe the relationship between test temperature and heart beat rates of individual embryos. These equations were then used to predict the heart beat rate of embryos at each incubation temperature. Data on the total duration of embryogenesis (=incubation period, from oviposition to hatching) of these species at different incubation temperatures were obtained from previous studies [*B. duperreyi* (Shine and Harlow, 1996); *T. septentrionalis* (Du, 2003); *P. sinensis* (Du and Ji, 2003; Ji et al., 2003)]. We calculated relative developmental rate for a given temperature by dividing the reported incubation duration at that temperature by the shortest incubation duration recorded for that species in the laboratory (25 days at 30°C for *B. duperreyi,* 23.5 days at 33°C for *T. septentrionalis* and 40 days at 33°C for *P. sinensis*) and taking the inverse of this value (Shine and Harlow, 1996). The ‘developmental zero’ of each species (the critical minimum temperature at which the rate of embryogenesis fell to zero) was then calculated from the linear relationship between developmental rate and incubation temperature (Shine and Harlow, 1996).

Eggs of many reptile species can tolerate temperatures well below the developmental zero [indeed, some can withstand temperatures close to freezing (Packard and Packard, 1988)]. Although embryonic growth ceases at such temperatures (Georges et al., 2005), the cardiovascular system of the animals continues to function [e.g. we have detected a heart beat of *B. duperreyi* embryos at 11°C (W.-G.D. and R.S., unpublished data)] and presumably this continued metabolic effort plays an important role in maintaining embryo viability. Accordingly, we defined two ‘cumulative heart beat’ parameters, based on either including or excluding heart beats occurring at temperatures below the developmental zero. The first parameter was the total number of heart beats (THB) of an embryo throughout its embryonic development, calculated at any given incubation temperature using the formula: THB = temperature-specific heart rate × total minutes of developmental time (= from oviposition to hatching) of eggs incubated at that temperature. Secondly, the total number of effective heart beats (TEHB) was calculated by subtracting heart rate at developmental zero from the formula, i.e. TEHB = (heart rate at that temperature – heart rate at the developmental zero) × total minutes of developmental time. The heart rate at developmental zero was estimated from the equation describing the relationship between heart rate and temperature within each species. This second parameter thus focuses only on heart beats likely to contribute to embryogenesis, i.e. those above the basal heart beat rate at which embryos survive but do not develop.

Statistical analysis

We used the software package of STATISTICS 6.0 to analyse data. Normality of distributions and homogeneity of variances were tested using the Kolmogorov–Smirnov test and Bartlett's test, respectively. One-way analysis of variances (ANOVAs) were conducted to test for the influence of incubation temperature on the total number of heart beats and the total number of effective heart beats, and Tukey's *post-hoc* multiple comparisons were used to distinguish among mean values of heart beats at each incubation temperature.

RESULTS

Mean egg masses for *B. duperreyi*, *T. septentrionalis* and *P. sinensis* were 0.447±0.017 g (±s.e.m.), 0.271±0.009 g (±s.e.m.) and 4.738±0.166 g (±s.e.m.), respectively. In all three species, higher incubation temperatures resulted in faster rates of embryonic heart beat (Fig. 1A), shorter total incubation periods (Fig. 1B) and higher developmental rates (Fig. 1C). Based on these linear relationships between developmental rate and incubation temperature, we estimated developmental zero temperatures (minimum for embryogenesis) as 14.2°C for both lizards (*B. duperreyi* and *T. septentrionalis*) and 16.0°C for the turtle (*P. sinensis*) (Fig. 1C).

In all three species, statistical analysis showed that the total number of heart beats over the course of incubation differed significantly among incubation temperatures (*P. sinensis*: $F_{4,170}$=32.75, P<0.0001; *T. septentrionalis*: $F_{4,65}$=53.10, P<0.0001; *B. duperreyi*: $F_{5,150}$=29.56, P<0.0001). Embryos that were incubated at low temperatures completed more heart beats prior to hatching than did conspecific animals incubated at higher temperatures. The total number of heart beats over the course of incubation increased considerably for embryos developing at temperatures below 26°C in *B. duperreyi* and *T. septentrionalis* and at temperatures below 28°C in *P. sinensis* (Fig. 2). By contrast, the total number of effective heart beats over the course of incubation was similar across incubation temperatures in all species (*P. sinensis*: $F_{4,170}$=2.14, P=0.08; *T. septentrionalis*: $F_{4,65}$=1.68, P=0.16; *B. duperreyi*: $F_{5,150}$=1.01, P=0.42) (see Fig. 2).

The total number of heart beats required to complete embryonic development at a standard incubation temperature (30°C) differed significantly among the three species ($F_{2,72}$=344.78, P<0.001), with *P. sinensis* requiring more heart beats over the course of incubation (6.72×10^6 *vs* 4.86×10^6 for *B. duperreyi* and 4.79×10^6 for *T. septentrionalis*). A similar interspecific difference was evident for the total number of effective heart beats ($F_{2,72}$=252.58, P<0.001), which averaged 5.56×10^6 for *P. sinensis*, 4.04×10^6 for *B. duperreyi* and 3.98×10^6 for *T. septentrionalis*.

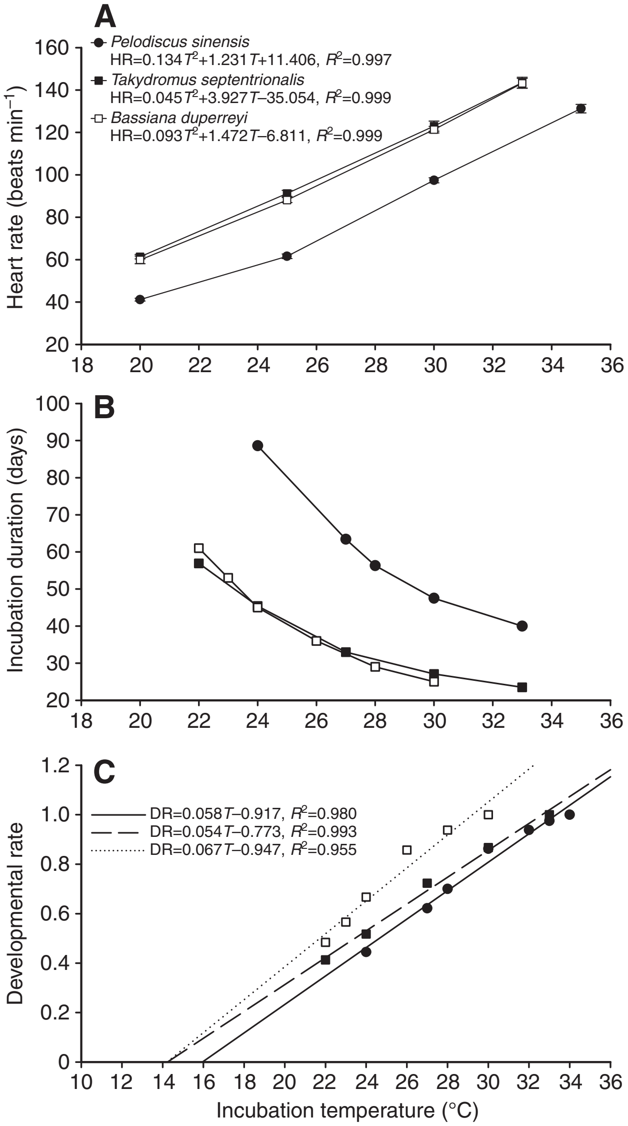

Fig. 1. Effects of mean incubation temperature on (A) heart beat rates (beats min^{-1}), (B) total duration of incubation and (C) developmental rates of the turtle *Pelodiscus sinensis* and the lizards *Takydromus septentrionalis* and *Bassiana duperreyi*. (A) Heart beat rates were measured by non-invasive methods and are expressed as means ± s.e.m. (B) Mean incubation durations were collected from published reports on *B. duperreyi* (Shine and Harlow, 1996), *T. septentrionalis* (Du, 2003) and *P. sinensis* (Du and Ji, 2003; Ji et al., 2003). (C) Developmental rate at each temperature was calculated by dividing incubation duration by the shortest incubation duration recorded in the laboratory and taking the inverse of this value. Data on *T. septentrionalis* and *P. sinensis* were taken from Du (Du, 2003) and Du and Ji (Du and Ji, 2003), respectively. Data on *B. duperreyi* were taken from Shine and Harlow (Shine and Harlow, 1996). HR, heart rate; *T*, temperature; DR, developmental rate.

DISCUSSION

Although the embryos of the three species were probably at different developmental stages at the time we monitored their heart beat rates, ontogenetic changes in heart rates in these species are so minor that they would have little or no impact on our calculations (Radder and Shine, 2006) (W.-G.D. and R.S., unpublished data). Our measurements of heart beat rates in lizard and turtle embryos reveal a broad similarity to the results of previous studies on birds in terms of the absolute number of heart beats required to complete embryogenesis [5 to 6×10^6 in the two lizard species, 8×10^6 in the turtle species (Fig. 2) and approximately 6 to 13×10^6 in birds (Ar and Tazawa, 1999)]. The total number of heart beats may, however, span a wider range in some other vertebrate lineages, such as mammals [e.g. human embryos take more than 6×10^7 heart beats to complete embryogenesis whereas mouse embryos take about 5×10^6 heart beats: calculated from data in Meier et al. (Meier et al., 1983)]. Lizards and turtles also resemble other taxa in showing a broad constancy of total heart beat numbers within embryos of a single species (Fig. 2). That relative constancy applies despite a twofold variation in total incubation duration in the non-avian reptiles we studied, a variation driven by incubation temperature (Fig. 1B).

The numbers quoted above actually underestimate the degree of similarity between birds, turtles and lizards in total number of heart beats during incubation. Both turtles and birds lay eggs with relatively undeveloped embryos, so that the period from oviposition to hatching encompasses all of embryogenesis (Booth and

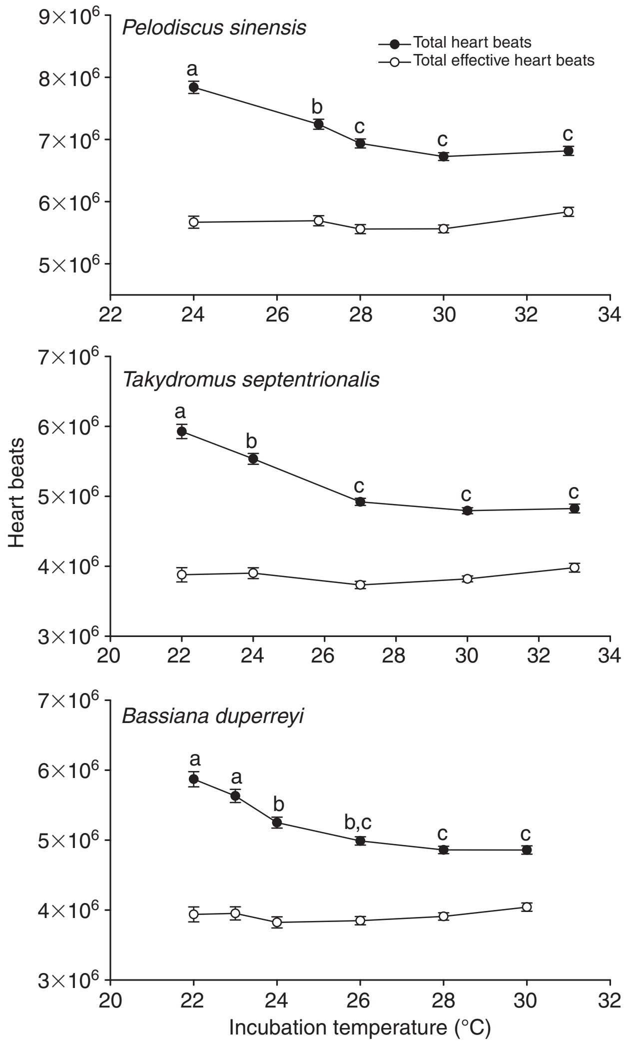

Fig. 2. Thermal dependence of the total number of heart beats prior to hatching and the total number of effective heart beats (i.e. excluding those that occur at temperatures too low for embryonic growth and differentiation) in the non-avian reptiles *Pelodiscus sinensis*, *Takydromus septentrionalis* and *Bassiana duperreyi*. Total heart beats over the course of incubation increased at lower temperatures whereas the total number of effective heart beats did not differ significantly among temperatures. Data are expressed as means ± s.e.m. Means with different letters above their error bars are statistically different (Tukey's *post-hoc* test).

Thompson, 1991; Andrews, 2004). By contrast, most squamates (including the two lizard species that we studied) delay oviposition until the embryo is partway (typically around 25%) through the total period of embryonic development (Shine, 1983; Andrews, 2004); and, thus, the number of heart beats estimated for lizards over the period from oviposition to hatching is substantially less than the total number over the entire course of embryogenesis. It would be of great interest to study lizards that lay their eggs at much earlier stages of embryonic development, as in some chameleons (Andrews et al., 2008) and to determine whether the inclusion of this 'missing' fraction (pre-laying) brings the sum heart beat count for lizards up to the same level as in birds and turtles.

Despite these similarities, the total number of heart beats for embryonic development clearly is not a fixed number and is affected both by developmental factors (such as the degree of offspring development at laying and at hatching) and by ecological factors (such as nest temperature). For example, the offspring of altricial birds hatch after fewer heart beats than do the offspring of precocial species (Ar and Tazawa, 1999; Tazawa et al., 2001). Similarly, egg size strongly affects heart beat rates in birds (Ar and Tazawa, 1999) and developmental time in many organisms (Gillooly et al., 2002). In non-avian reptiles, thermal conditions in the nest will probably be the most important proximate factor affecting both heart rates and the duration of incubation. Laboratory experiments generally have reported little to no effect of hydric conditions on incubation duration (e.g. Flatt et al., 2001; Ji and Du, 2001; Booth, 2002) or heart rates (Du and Shine, 2008) whereas temperature strongly affects both of these variables (Birchard and Deeming, 2004; Radder and Shine, 2006) (see Fig. 1A and Fig. 1B). The thermal dependencies of embryogenesis and of heart rates effectively cancel each other out over a range of warm conditions, i.e. development accelerates with temperature as the same rate as heart beat, thus maintaining an approximate constancy in total heart beats prior to hatching (Fig. 2). That equivalence disappears at lower temperatures, however, such that cool-incubated embryos complete more heart beats prior to hatching than do their warm-incubated siblings (Fig. 2). Although thermal acclimation might affect the rate of embryonic heart beats [as found in the snapping turtle, *Chelydra serpentina* (Birchard and Reiber, 1996)], most studies on this topic have concluded that the growth efficiencies and energetics of reptilian embryos show little evidence of thermal acclimation (Whitehead et al., 1992; Angilletta et al., 2006).

We attribute the increase in cumulative number of heart beats at lower temperatures to a thermally-driven shift in the magnitude of maintenance costs relative to the cardiovascular effort devoted to 'productive' embryogenesis. At the developmental zero temperature, heart beat rates decline but do not cease. Under these conditions, all of the embryo's metabolic work is directed to maintenance. Below the developmental zero temperature, the embryo continues to respire (and thus its heart continues to beat, albeit slowly) but it does not grow or differentiate. Thus, for example, an embryo exposed to a fluctuating thermal regime that rises above developmental zero for only a few hours per day will necessarily accumulate a large number of 'maintenance' heart beats that achieve little or nothing towards furthering its ontogenetic development. By the end of incubation, such an embryo will thus have completed many more heart beats, in absolute terms, than a warm-incubated sibling. This interpretation is supported by the constancy of 'cumulative number of effective heart beats' across a wide range of incubation conditions, in contrast to the thermal dependence of total number of heart beats (Fig. 2). These results support and extend ideas regarding a constancy in the number of degree-days needed to complete the developmental process (Trudgill et al., 2005). The concept of the total effective heart beat provides a functional explanation for the relationship between incubation temperature and developmental time.

Why don't bird eggs show similar thermal dependence in total numbers of heart beats? They may well do so but any thermal effect is masked by the fact that: (1) maternal brooding reduces the variance in thermal regimes during incubation, both among eggs within a clutch and among clutches; and (2) temperature may have little effect on heart beat rate within the bird's thermoneutral zone (Ar and Tazawa, 1999; Tazawa, 2005). Non-avian reptiles experience different conditions. Many embryos of oviparous reptiles develop in shallow nests that display wide thermal fluctuations on a diel and/or seasonal cycle (Shine et al., 1997; Ackerman and Lott, 2004; Shine, 2004; Du and Feng, 2008). Accordingly, embryos often experience temperatures below the developmental zero for considerable periods of time. For example, developmental zero is 14.2°C in *T. septentrionalis* and *B. duperreyi* but temperatures in natural nests have been recorded to fall to 11.6°C for *T. septentrionalis* (Du and Ji, 2006) and 9°C for *B. duperreyi* (Shine and Harlow, 1996). Because they experience a more variable and less predictable thermal environment (unlike maternally-brooded bird eggs), squamate eggs thus must be able to adjust their total cardiovascular effort flexibly to conditions in the nest.

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